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Random Cross-Species Identification of a Gene Encoding for an Inorganic Pyrophosphatase in the Uncultivable Hemotrophic Bacterium *Mycoplasma suis*

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II Summary

Genome sequence analysis of *Mycoplasma suis* is hampered due to the lack of an appropriate in vitro cultivating system. The organism has still to be purified from blood samples of experimentally infected pigs, which is linked with technical difficulties. Therefore, little is known about the genome of *M. suis*. For this work a cross-species random approach, based upon Southern blot, was considered in order to identify novel genes. Two shotgun *M. suis* genomic libraries were screened using either *M. pneumoniae* enolase or pyruvate dehydrogenase (PDH) probes. Using these two enzymes as probes is based on recent gained insights of their pathogenicity in mycoplasma infections. Following this strategy the complete open reading frame of a putative *M. suis* inorganic pyrophosphatase (ppA) was found by hybridizising with a *M. pneumoniae* enolase probe. The positive hybridization signal is considered to result from the DNA homology of 45% between the *M. suis* ppA and the *M. pneumoniae* enolase. The encoding gene ppA is 495 bp in size. The deduced amino acid sequence showed an overall similarity of 53 to 55% with inorganic pyrophosphatases of other pathogenic mycoplasmas, and a GC-content of 32%. This study demonstrates the feasibility and the encountered difficulties of a random cross-species approach to identify novel genes of an uncultivable agent like *M. suis*.

1 Introduction

1.1 Biological features of *Mycoplasma suis*

Mycoplasma (*M.*) *suis* belongs to the group of uncultivable hemotrophic mycoplasmas that parasitize the red blood cells (RBC) of a wide range of vertebrates with a pronounced species specificity (Messick, 2004). In pigs, the resulting disease, referred to as porcine Eperythrozoonosis (PE), is worldwide distributed causing appreciable economic losses in pig industry.

Hemotrophic mycoplasmas belong to the genus *Mycoplasma* due to their phylogenetic relatedness (sequence analysis of the 16S rDNA) and phenotypic characteristics (lack of intracellular parasitism, small size, lack of a cell wall, lack of flagellae, resistance to penicillin, and susceptibility to tetracyclines; Messick et al., 2002; Neimark et al., 2002; Neimark et al., 2001). Within the genus *Mycoplasma* this group of uncultivable RBC pathogens and organisms belong to the *pneumoniae* group of mycoplasmas and represent a distinct new cluster and are referred to as Hemoplasmas. (Neimark et al., 2002, 2001; Figure 1).

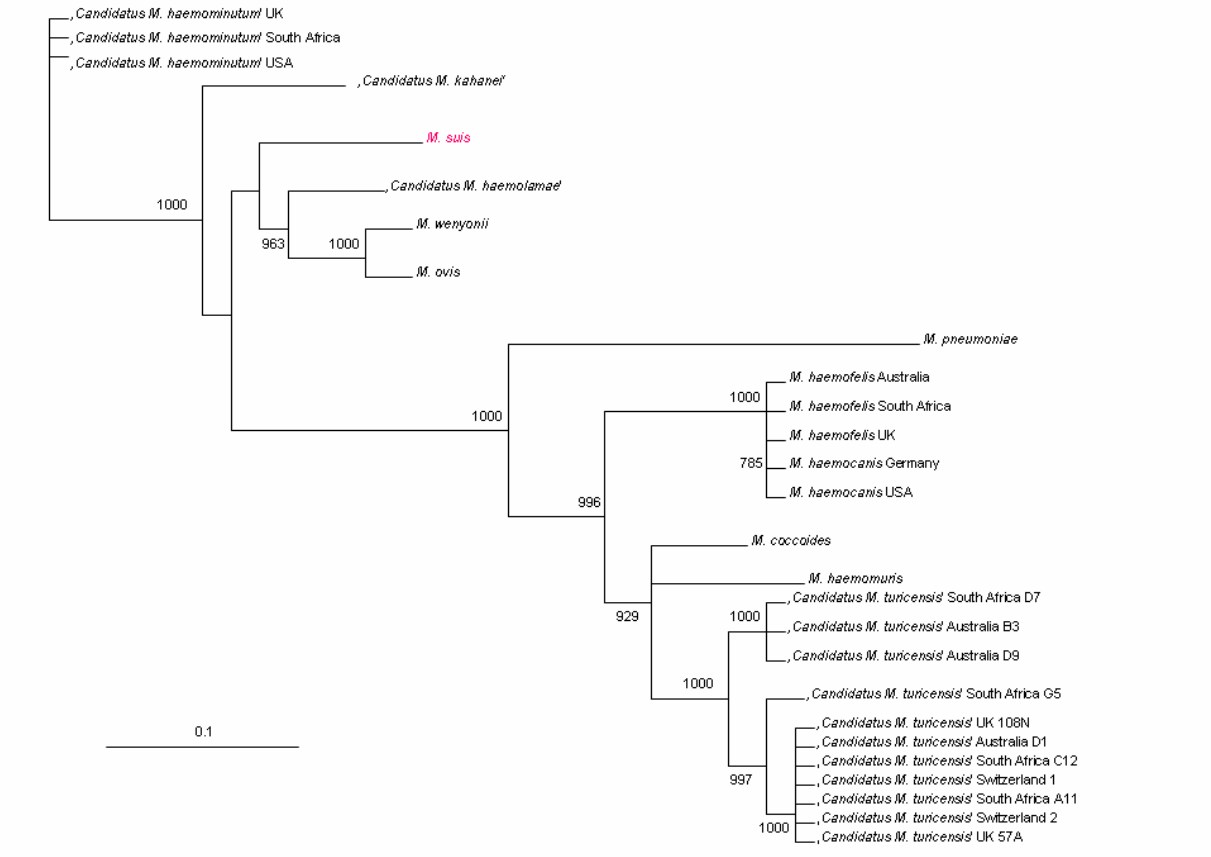

Figure 1. Phylogenetic analysis of 16S rDNA sequences (Willi et al., 2006).

Hemoplasmas are cell wall less, pleomorphic bacteria (rod-shaped, spherical, or ring-shaped) and are found individually or in chains across the red cell surface. Their size ranges from 375 to 600 nm in diameter (Messick, 2004; Zachary and Basgall, 1985; Pospischil and Hoffmann, 1982). Like all hemotrophic organisms, *M. suis* stains blue to purple with the Wright-Giemsa's stain and it is visible on the erythrocyte surface after acridine orange staining where it is found individually or in chains (Figure 2). The organisms are also found free in the plasma (Neimark et al., 2001; Zachary and Basgall, 1985).

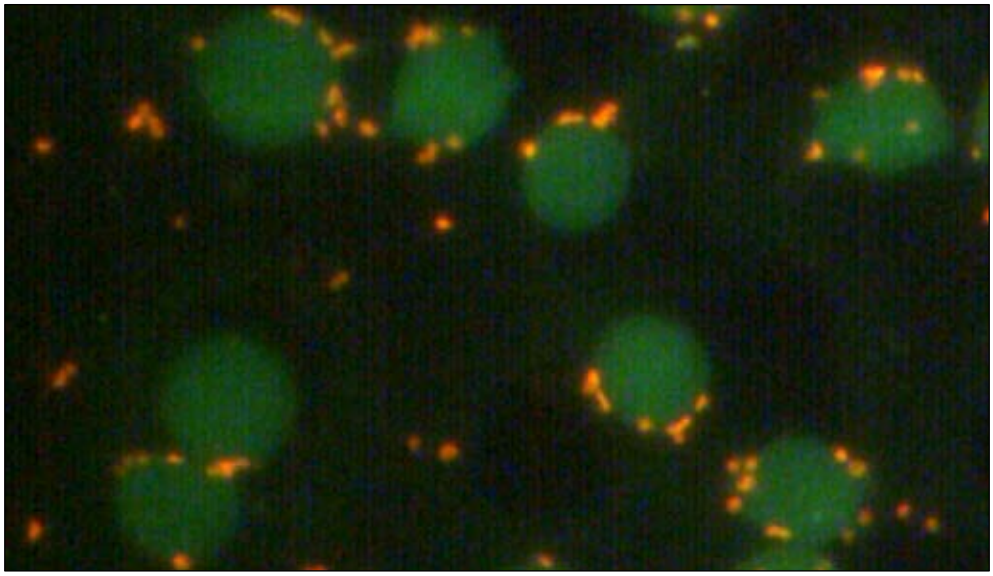

Figure 2. Acridin orange stained blood smear from an experimentally *M. suis* infected pig during maximum bacteremia. *M. suis* cells are visible as orange stained particles on the surface of RBCs (Hoelzle et al., 2007b).

Hemoplasmas parasitize on the surface of RBCs in a unique life-cycle and it is evident that these agents also replicate on RBCs. For *M. suis*, electron microscopic investigations demonstrated at least four different replication forms (small coccoid immature form, juvenile discoid form, mature ring-shaped form) on the RBC surface indicating that *M. suis* multiplies on the RBC surface (Zachary and Basgall, 1985). The contact between host cell and pathogen is rather intimately as shown in several electron microscopic investigations (Zachary and Basgall, 1985; Pospischil and Hoffmann, 1982). This interaction observed in vivo is accompanied by a significant deformation of the RBC surface with prominent pits, trenches, and invaginations.

Within these deformed membrane areas, *M*. *suis* organisms are intimately associated with but distinctly separated from the membrane by a 30-nm electron lucent zone. In this zone, fine fibrillar attachments between *M*. *suis* organism and the host cell membrane could be observed.

M. suis possesses a circular, double-stranded DNA with a genomic size of 745 kb (Messick, 2004). This relatively small genome ranges within the published sizes (580 to 1400 kb) for the pneumoniae subgroup of mycoplasmas. Their DNA molecules mainly provide the genetic information for replication, transcription and protein synthesis (Messick, 2004). Other genes e.g. the genetic basis to synthesize cell wall components (peptidoglycan) have been lost (Woese, 1987). All mycoplasma genomes have a characteristically low G+C content within the range of 24 to 33 mol% (Razin et al., 1998). Furthermore, an alternative genetic code is used by all members of the Genus *Mycoplasma*, where the codon UGA encodes the amino acid tryptophan instead of the common stop signal (Razin et al., 1998).

1.2 Porcine Eperythrozoonosis

1.2.1 Disease

M. suis infections are prevalent worldwide and are of serious economical significance. Pigs of any age can be affected by *M. suis* infections. For acute affections determining factors i.e. weaning, birth, periparturient period, sickness, viral infections, and other types of stress seem to be predisposing (Gresham et al., 1994; Heinritzi, 1990).

Acute *M. suis* infections are characterized by pallor, fever up to 42 °C, icteroanemia, and cyanosis in the extremities, mostly on the margins of the ear cartilage (Figure 2). This acute phase of disease is associated with massive bacteremia, causing severe and sometimes lethal hemolytic anemia (Messick, 2004; Zachary and Basgall, 1985). During the clinical acute PE, a radical decrease of the blood glucose concentration is observed leading to a life-threatening hypoglycemia (Gwaltney and Oberst, 1994; Heinritzi et al., 1984). Growth rate decreases in pigs that survive the acute phase of the disease. In sows, infection may lead to pyrexia, anorexia, depression, decreased milk production and poor maternal behaviour. (Heinritzi, 1992; Zinn et al., 1983; Brownback, 1981; Henry, 1979). In experimentally infected acute diseased pigs early and late hematological alterations are observed (Zachary and Smith, 1985). Early hematological alterations are massive parasitism of RBCs, a severe hypoglycemia, a

moderate bilirubinemia, and a mild anemia. Later findings include a severe anemia, minimal parasitism of RBCs, a spontaneous agglutination of RBCs at 25°C and 4°C, a transient thrombocytopenia and a mild bilirubinemia.

Figure 3. Piglet suffering from anemia, hypoglycemia and acrocyanosis.

Nowadays, there has been a marked decrease in incidence of the acute form of PE since antibiotic treatment of pigs e.g. with tetracycline via feeding is available as a therapeutic measure. Nonetheless, pigs still may become persistently infected asymptomatic carriers (Messick, 2004; Smith, 1992; Heinritzi, 1990).

Chronic low-grade *M. suis* infections are of economical significance and appear either as asymptomatic infections or wide ranging complexes of symptoms i.e. unthriftiness, pallor, and sometimes allergic skin reactions in form of urticaria or Morbus maculosus, poor reproductive performance in sows such as poor conception rates, irregular cycles, anestrus, abortion, small litters, premature births, stillbirths and the birth of weak piglets (Schweighardt et al., 1986; Zinn et al., 1983; Brownback, 1981; Henry, 1979).

Of high significance is the fact that chronic *M. suis* infection suppresses the host's immune system which leads to an increased susceptibility to infectious agents of the respiratory and intestinal tract. In spite of an intense immune response of the host during an acute *M. suis* infection and even after antibiotic treatment the host is not

able to eliminate the agent. *M. suis* can persist for years in the asymptomatic carrier animals without any detectable clinical symptoms and an acute *M. suis* attack is likely at any time e.g. under bad body conditions or stress. These carriers are important regarding the eradication and epidemiology of *M. suis* infections.

1.2.2 Epidemiology

Transmission of *M. suis* can occur directly via the oral uptake of blood and blood components: licking tail-docking wounds, cannibalism, or the uptake of blood-contaminated urine. There is also an indirect transmission by means of living vectors, such as ectoparasites, and nonliving vectors, such as contaminated surgical instruments used for tattooing or castration (Heinritzi, 1992).

1.2.3 Pathogenesis

To date virulence determinants of *M. suis* could not be elucidated in detail mainly due to the lack of an in vitro cultivation system. Thus, the recent knowledge on pathogenic phenomenons during acute PE are based on rather limited in vitro experiments e.g. on the apparently *M. suis*-induced glycolytic activities in infected blood, and on a series of experimental infections in pigs.

As mentioned above, *M. suis* is in close contact with the RBC membrane (Zachary and Basgall, 1985; Pospischil and Hoffmann, 1982). This interaction causes deformations and modifications of the RBC membrane, which leads to an induction of auto-reactive IgM antibodies, so called cold agglutinins (CA). The attachment of CAs to the RBCs results in an immune-mediated severe hemolytic anemia in acute ill pigs (Jüngling et al., 1994; Zachary and Smith, 1985). This phase of anemia goes along with a strong decrease of the agent in blood. The biological activities of CA are considered to be directly responsible for clinical symptoms in pigs suffering from PE such as acrocyanosis and pallor and probably also for the suppressive effect on T-lymphocyte blastogenic responses (Zachary and Smith, 1985). However, the exact attachment mechanism of *M. suis* leading to the close contact with the host cell RBC and the pathogen is still unknown. Very recently, Hoelzle and coworkers (2007b) identified for the first time a *M. suis* protein, designated MSG1 (*M. suis* GAPDH-homologous protein 1), which apparently mediates adhesion of *M. suis* cells to the RBC surface. Further studies are required to identify other membrane interactors that

could be part of a potential adhesion complex of *M. suis,* and to identify erythrocyte receptors that bind putative *M. suis* adhesins.

Acute *M. suis* infection produces a serious blood acidosis and hypoglycemia (Heinritzi, 1989). During an acute attack of PE the blood glucose consumption increases strongly leading to a life-threatening hypoglycemia. This hypoglycemia could be strongly associated with the metabolic activities of *M. suis* (Heinritzi et al, 1990; Smith et al., 1990). The acidosis results from an increase in the lactic acid concentration, as well as from a disturbance in the pulmonary gas exchange (Henry, 1979).

The immunological response of pigs against *M. suis* is characterized amongst others by induction of anti-erythrocyte CAs (Schmidt et al., 1992; Zachary and Smith, 1985). CAs are directed against carbohydrate antigens expressed on the erythrocyte surface and not against *M. suis* antigens itself (Havouis et al., 2002; Potter, 2000; Jüngling et al., 1994; Schmidt et al., 1992; Zachary and Smith, 1985). Nevertheless, the precise role of CAs in the pathogenesis of PE is not yet clear.

Recently, a detailed analysis of the humoral immune response during experimental infection resulted in the identification of at least eight *M. suis* specific antigens which are targeted by specific IgG antibodies (Hoelzle et al., 2006). Three proteins, i.e. p40, p45, and p70 were the preferentially recognized *M. suis* antigens. This IgG immune response was observed in all infected pigs by 14 days post infection at the latest and until week 14, the end of the experiments. Interestingly, in this study, a striking IgG based autoimmune response was found during acute clinical attacks of eperythrozoonosis which is accompanied by a derailment of the *M. suis* specific antibody response. The exact mechanisms of this pathoimmunologic feature and the porcine targets of the IgG autoantibodies remains unclear so far, and are, at the moment, under investigation. Recently, the nature and identity of two of the three major *M. suis* antigens could be elucidated by applying serological proteome analysis in conjunction with mass spectrometry methods and screening of genomic *M. suis* libraries (Hoelzle et al., 2006, 2007a). The p70 antigen was identified as a heat shock protein HspA 1 and p40 as GAPDH-homologous protein MSG1. Both proteins could be localized in the cytoplasm as well as in the membrane and on the surface of *M. suis* indicating a role in the pathogenesis of PE.

1.2.4 Diagnosis

Diagnosis of *M. suis* infections is still a challenge due to the lack of pure culture as a crucial diagnostic measure and as a source of purified diagnostic antigens or DNA to establish a reliable serology or DNA amplification techniques, respectively. Therefore, laboratory diagnosis often relies on the microscopic examination of chemically stained peripheral blood smears to directly visualize the microorganisms attached to RBC. The application of PCR methods has increased the specificity and sensitivity of the *M. suis* diagnostics (Hoelzle et al., 2003; Messick et al., 2000; Oberst et al., 1993).

Serological diagnostics are often the methods of choice. The application of serological tests would allow cost-effective diagnosis on herd basis, follow-up investigations, and extensive prevalence studies. However, the routine application of serological assays for the serodiagnosis of *M. suis* is difficult. Two circumstances, namely the antigen preparation from the blood of experimentally infected animals that is marked by extreme variability among batches and the immune globulin impurities of the *M. suis* antigens (Hoelzle et al., 2006) obstructs the accurate adoption and standardization of serodiagnostic techniques.

Recently, two major immunogenic proteins (p40 and p70; Hoelzle et al., 2006, 2007a, b) could be produced in vitro using a heterologous *Escherichia coli* expression system (Hoelzle et al., 2006, 2007a,b) and are now available for establishing standardized and reproducible tools for routine diagnostic procedures as well as for investigating systematic analyzes of the biology of *M. suis* (Hoelzle et al., 2007a,b).

2 Thesis concept

Our group has recently performed serological proteom analyses of *M. suis* antigens which revealed eleven immunoreactive proteins. One protein was identified by PMF-MALDI-TOF as enolase and one protein as pyruvate dehydrogenase (Hoelze et al., 2006). We assumed that both proteins could play an important role in the pathogenesis of porcine *M. suis* infections since pathogenic features of enolase and pyruvate dehydrogenase are described for other bacterial pathogens. Enolase, a key glycolytic enzyme, belongs to a class of multifunctional surface proteins with binding properties. For example, enolase of group A streptococci is described as a

plasminogen binding receptor and enolase of staphylococci binds to laminin (Pancholi, 2001). Furthermore, in *M. pneumoniae,* pyruvate dehydrogenase is part of the adhesion complex which mediates the attachment to mucosal cells (Dallo et al., 2002).

The aim of this work was to investigate whether *M. suis* features both, the genes encoding enolase and pyruvate dehyrogenase. Since *M. suis* is not cultivable and the *M. suis* genome is not sequenced so far, we applied a cross-species identification approach: two *M. suis* genomic DNA libraries SG1 and SG2 were screened using DNA probes specific for *M. pneumoniae*, a closely related organism with similar biological properties i.e. cold agglutinin induction and adhesion to erythrocytes. Hybridizing clones were sequenced and analyzed to get evidence of the nature and function of the reacting clones.

3 Material and Methods

3.1 Bacterial strains and plasmids

M. suis strain 54/96 was maintained in splenectomized piglets by repeated artificial infections as described by Hoelzle et al. (2003). *E. coli* K12 DH5α and Top10 strains (Invitrogen, Basel, Switzerland) were grown in Luria-Bertani (LB) broth (Merck, Darmstadt, Germany), used to construct the *M. suis* genomic library and to clone *M. suis* DNA fragments. The vector pUC 18/19 (Roche, Rotkreuz, Switzerland) was chosen for DNA manipulations. Its multiple cloning site is located within the *lacZ* (β-galactosidase) gene. The cloning of inserts results in the cleavage of the β-galactosidase activity allowing blue/white selection. The *E. coli* ampicillin resistance gene and origin of replication elements allow for easy plasmid amplification and selection (Figure 3).

3.2 DNA extraction and library construction

M. suis cells were purified from the blood of experimentally infected pigs by using a differentiating centrifugation method (Hoelzle et al., 2003). For DNA extraction, the *M. suis* preparation was incubated overnight at 37°C in lysis buffer (120 mM NaCl, 10 mM EDTA, 25 mM Tris, pH 7.4, 1% N-lauroylsarcosine) containing 1 mg of proteinase K (Roche) per ml. DNA was isolated by phenol-chloroform extraction and

ethanol precipitation (Hoelzle et al., 2003). Porcine DNA from the blood of *M. suis* negative pigs was prepared accordingly.

M. suis DNA library construction was performed by Medigenomix (Martinsried, Germany). Briefly, a total of 10 μg *M. suis* DNA was sonicated. After fragmentation the DNA was end-filled using Klenow fragment (Roche). Medium-sized fragments of about 1.5 kb (SG1) and from 2.5 to 3.0 kb (SG2) were ligated into a blunt-end cut pUC19 vector (*Sma*I digested) and transformed into *E. coli* K12 Dh5α.

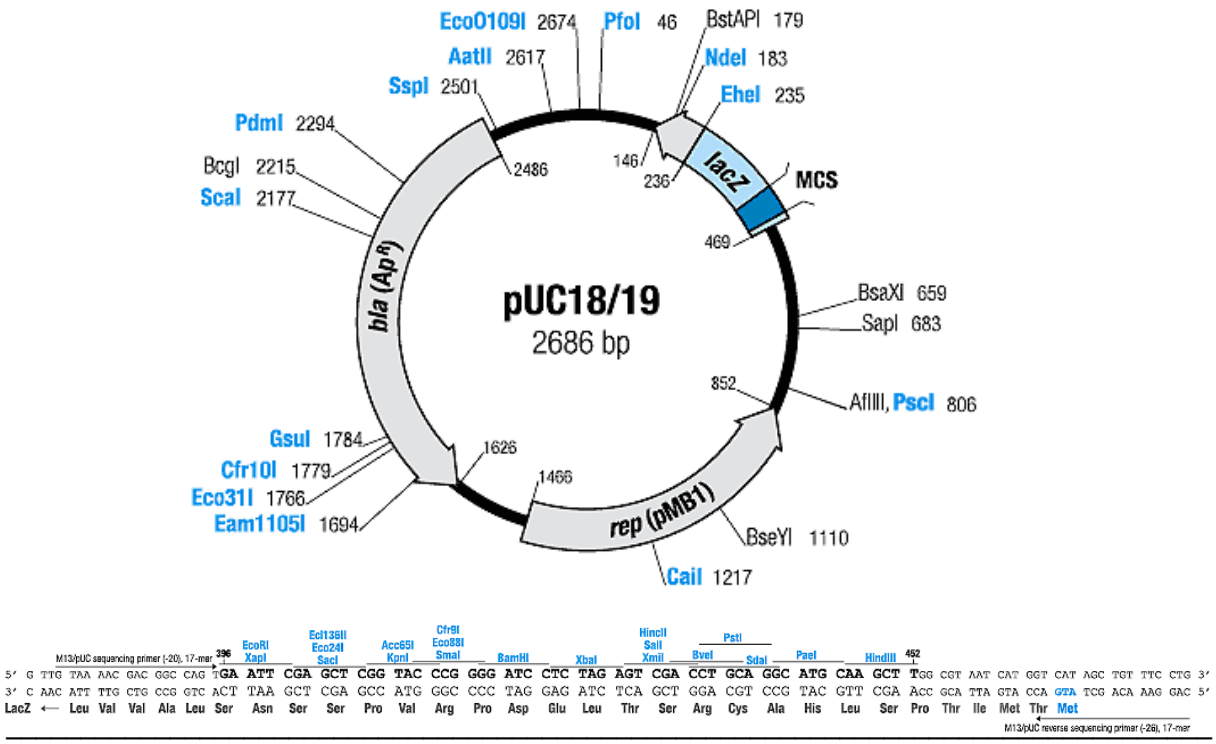

Figure 4. The pUC 18/19 was used for the construction of the *M. suis* genomic library and the cloning of *M. suis* genome fragments. MCS: multiple cloning site; *bla*: ampicillin resistance gene; *rep*: origin of replication.

3.3 Plasmid purification (alkaline lysis)

1.5 ml of overnight *E. coli* cultures were centrifuged at 6000 rpm for 5 min at ambient temperature. The supernatant was discarded and the pellet resuspended in 300 μl of buffer I (50 mM Tris, pH 8.0, 10 mM EDTA). Then, 300 μl of buffer II (0.2 M NaOH, 1% SDS) was added to lyse the *E. coli* cells. Suspensions were mixed by gentle inversion and incubated for 5 min at ambient temperature. Then, 300 μl of buffer III (3 M potassium acetate, 2 M acetic acid) was added and the mixture was placed on ice

for 15 min. Afterwards the samples were centrifuged at 15'000 rpm for 20 min at 4°C. 800 µl of the supernatant were carefully transferred into new reaction tubes avoiding transferring the white debris. The plasmid DNA was precipitated by adding 800 µl of ethanol (100%, ambient temperature) and centrifugation at 15'000 rpm for 30 min at 4°C. Subsequently, the supernatant was discarded and the DNA pellet was air dried. H_2O (supplemented with 10 µg/ml RNase) was added to resolve the plasmid DNA.

3.4 Preparation of DNA probes

3.4.1 Digoxigenin (DIG) labeled DNA probes

PCR amplicons of the *M. pneumoniae enolase* and *pyruvate dehydrogenase* (*pdh*) genes as well as recombinant plasmid insert-DNA were used to prepare DIG labeled DNA probes. Recombinant plasmid DNA was extracted from 50 ml overnight *E. coli* cultures using the GenElute HP Midiprep Kit (Sigma) following the manufacturer's instructions. Purified plasmid DNA was digested with *Eco*RI. The insert DNA fragments and PCR amplicons were isolated from the agarose gel and purified with the QIAquick Gel Extraction Kit (Qiagen, Hombrechtikon, Switzerland) following the manufacturer's instructions.

For the labeling reaction the DIG DNA Labeling and Detection Kit (Roche) was used. The concentration of DNA was determined spectrophotometrically at 260 nm (BioPhotometer, Vaudaux-Eppendorf, Schönenbuch, Switzerland) and adjusted to 1 µg in 15 µl H_2O. Then, the DNA was denaturated at 95°C for 10 min and immediately placed on ice. Hexanucleotide mixture (2 µl), dNTP mixture (2 µl) and Klenow fragment (1 µl) were added and the reaction mixture was incubated for 19 h at 37°C. The reaction was stopped by adding 2 µl of 0.2 M EDTA. Before use DIG labeled probes were denatured (95°C, 10 min) and diluted in 13 ml DIG Easy Hyb (Roche) hybridization buffer.

3.4.2 Biotinylated porcine DNA probes

For colony blot hybridization total porcine DNA was derived from the blood of healthy SPF pigs and was biotin labeled using the protocol Round A/B/C random amplification of DNA (DeRisi Lab, UC San Francisco, June 2001, adapted from Bohlander et al., 1992). Before use the biotin labeled probe was denatured (95°C, 10 min) and diluted 1:50 in 3xDNA hybridization buffer (Clondiag, Jena, Germany).

3.5 Colony blot hybridization

The colony blot hybridization technique was used to select out library clones containing porcine DNA inserts. Ten fold dilutions of the *M. suis* library were plated onto LB agar plates containing 100 µg/ml ampicillin and incubated overnight at 37°C. A positively charged membrane disc (Roche) was placed on the surface of the library LB agar plates with approximately 100 *E. coli* colonies. After 1 min the membrane was gently removed from the surface and placed for 15 min colony side-up in a glass dish containing two filter papers soaked with denaturation solution (0.5 M NaOH, 1.5 M NaCl). Then, the membrane was laid for 15 min onto two filter papers soaked with neutralizing solution (1.0 M Tris, 1.5 M NaCl, pH 7.5). Afterwards the membrane was placed for 10 min onto two filter papers soaked with 2xSSC buffer (0.3 M NaCl, 30 mM Natriumcitrat, pH 7.0). The DNA on the membrane was fixed by means of UVcross-linking (0.15 Jcm^{-2} for 120 s) using a BIO-LINK® crosslinker, type BLX254 (LTF Labortechnik, Wasserburg, Germany). Subsequently the membrane was treated with proteinase K solution (2 mg/ml proteinase K [Roche] in 2xSSC buffer) and incubated for 1 h at 37°C. In order to remove the cell debris, the membrane was put between two filter papers soaked in distilled water and a pipette was rolled over with adequate pressure. Then, the membrane was prehybridized for 15 min at 50°C using 3 ml prehybridizing solution (1xSSC/1% bovine serum albumin).

For hybridization the membrane was incubated with the biotinylated porcine DNA probe for 1 h at 60°C. Then the membrane was washed with washing buffer 1 (2xSSC/0.01% Triton X-100) for 5 min at 30°C and with washing buffer 2 (2xSSC) and 3 (0.2xSSC) for 5 min each at 20°C. Thereafter, the membrane was equilibrated in maleic acid buffer (0.1 M maleic acid, 0.15 M NaCl, pH 7.5) and free binding positions were blocked by incubating the membrane in blocking buffer (1% blocking reagent [Roche] in maleic acid buffer) for 1 h at ambient temperature. The biotinylated probe target DNA hybrids were detected using alkaline phosphatase (AP) conjugated streptavidin diluted 1:2000 in blocking buffer for 30 min. Then, the membrane was washed twice with maleic acid buffer for 10 min. The development was performed using Nitrobluetetrazoliumchloride/5-Bromo-4-chloro-3-indolylphosphate (NBT/BCIP, Roche) diluted 1:50 in detection buffer (100 mM Tris, 100 mM NaCl, pH 9.5). AP cleaves the substrate 5-Bromo-4-chloro-3-indolylphosphate which is supplemented with the enhancer chromogen

Nitrobluetetrazoliumchloride. This results in the formation of an insoluble blue precipitate. To stop the reaction the membrane was placed in distilled water.

3.6 Southern blot hybridization

In order to identify *M. suis enolase* or *pdh* containing library clones Southern blot hybridization analysis of *M. suis* library clones was performed. Colony blot negative clones were picked from the LB agar plates. Recombinant plasmids were prepared by alkaline lysis and digested with *Eco*RI and *Hind*III (Roche) according to the manufacturer`s instructions. Briefly, 2.0 µl plasmid DNA (300 ng), 0.5 µl *Eco*RI, 0.5 µl *Hind*III, and 2.0 µl reaction buffer B were mixed with 15 µl H_2O and incubated at 37°C for 2h.

Agarose gel electrophoresis was used to separate the DNA fragments according to their sizes. 0.8 to 1% agarose (Eurobio, Chemie Brunschwig AG, Basel, Switzerland) in 1xTAE buffer (40 mM Tris 20 mM acetic acid, 1 mM EDTA) was mixed and melted in a microwave oven. Then ethidium bromide (Merck) was added (3 µl/100 ml 1xTAE). The melted agarose was poured onto a gel plate to a depth of 1 cm and cooled down for 30 min. The gel plate was submerged in 1xTAE buffer and the comb was removed. 6xloading dye (Biolabs, Frankfurt, Germany) was added to the sample and the digest was run for 40 min at 80 V (plasmid DNA) or overnight at 30 V (genomic DNA digest). The fragments obtained were analyzed under UV illumination (λ = 254 nm).

Agarose gels were blotted onto a nylon membrane (Hybond-N, Amersham Bioscience, Otelfingen, Switzerland) by alkaline capillary blotting in accordance to a standard procedure (Sambrook and Russell, 2001). First, the plasmid DNA on the agarose gel was denatured in 0.4 M NaOH for 10 min at ambient temperature. Then the denatured DNA was transferred overnight as shown in Figure 5.

After the transfer, the membranes were rinsed for 10 min in 2xSSC buffer followed by prehybridization at 37°C for 30 min in hybridization solution (DIG Easy Hyb, Roche). DNA was hybridized for 8–12 h at 37°C either with the DIG labeled *M. pneumoniae enolase* probe or the DIG labeled *M. pneumoniae pdh* probe. The membranes were washed at ambient temperature twice for 5 min in 2xSSC/0.1% SDS and at 62°C twice for 20 min in 0.1xSSC/0.1% SDS.

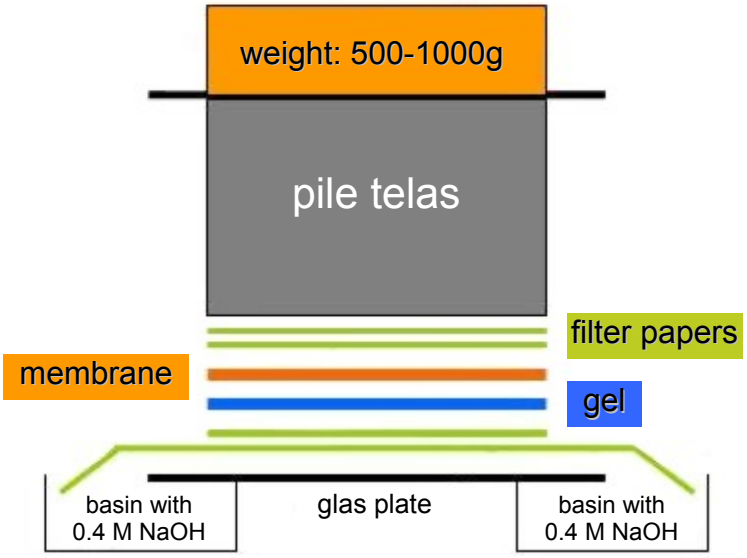

Figure 5. Southern blot: set up of capillary blotting.

Immunodetection was performed with an anti-DIG Fab fragment conjugated with alkaline phosphatase (Roche) by using the methods described in chapter 3.5 Colony blot hybridization.

To identify the *M. suis* genomic fragment carrying the *ppA* gene Southern blot hybridization of genomic *M. suis* DNA was performed using a DIG labeled *ppA*-probe. *M. suis* genomic DNA was digested with *Eco*RI: 2 µg *M. suis* DNA (15 µl) was mixed with 2.0 µl reaction buffer H, 1.0 µl *Eco*RI, and 2.0 µl H_2O and incubated at 37°C overnight. The genomic DNA digests were run on an 0.8% agarose gel overnight. Thereafter, the DNA was depurinated twice in 0.25 M HCl for 5 min, then washed trice in distilled water for 5 min and subsequently denatured in denaturing buffer (0.5 M NaOH 1.5 M NaCl). Transfer, hybridization procedure and immunological detection were performed as described above.

3.7 Cloning procedure

Positive hybridized bands were cut from a genomic *M. suis* DNA *Eco*RI digest on an 0.8% agarose gel. The DNA was purified with QIAquick Gel Extraction Kit (Qiagen) following the manufacturer's instruction.

For cloning the pUC 18 vector (Roche) was digested with *Eco*RI and treated with calf intestinal phosphatase (Roche) according to a standard protocol (Sambrook and

Russell, 2001) in order to dephosphorylate the 5' ends and to avoid religation of the linearized vector DNA.

For the ligation reaction vector and insert DNA were mixed in a molar ratio of 1:5 together with 1 µl T4 ligase (Roche), 2 µl T4 ligation buffer (10x), and distilled water to a final volume of 20 µl. The mixture was incubated overnight at 14°C.

For the transformation chemically competent *E. coli* Top10 cells were prepared according to the method of Hanahan (1983) with slight modifications. One ml of an *E. coli* overnight culture was used to inoculate 200 ml LB medium. The culture was incubated at 37°C until an OD_{660nm} of 0.4 - 0.55 was reached. The *E. coli* cells were cooled for 10 min on ice and centrifuged at 3000 rpm, 4°C for 10 min (Heraeus Sepatech, Suprafuge 22, rotor HFA 21.94). The pellet was resuspended in 4 ml TFB I solution (30 mM postassium acetate, 50 mM $MnCl_2$, 100 mM $RbCl_2$, 10 mM $CaCl_2$, 15% glycerin, pH 5.8). The suspension was incubated for 10 min on ice and centrifuged as described above. Finally, the pellet was resuspended in 4 ml TFB II solution (10 mM MOPS, 10 mM $RbCl_2$, 75 mM $CaCl_2$, 15% glycerin, pH 7.0), and 60-µl aliquots of the chemically competent *E. coli* Top 10 were stored at -80°C.

Transformation of the ligation mixture into competent *E. coli* was performed as follows: 2 µl ligated sample was added to 60 µl chemically competent *E. coli* and chilled on ice for 30 min. Afterwards bacteria were heat-shocked for exactly 1 min at 42°C in a water bath, and then 250 µl SOC medium (Invitrogen) was added. The suspension was incubated at 37°C for 1 h. To get single colonies *E. coli* were plated on LB agar plates supplemented with 100 µg/ml ampicillin, 80 µg/ml X-Gal (5-bromo-4-chloro-3-indolyl-ß-galactopyranoside, Biosolve), and 240 µg/ml IPTG (isopropyl-ß-D-thiogalactopyranoside, Biosolve) and incubated at 37°C overnight.

3.8 Polymerase chain reaction (PCR)

PCR amplification of the *M. pneumoniae enolase* and *pdh* genes as well as the *M. suis ppA* gene was performed using the HotStarTaq Polymerase Master Mix (Qiagen) and the oligonucleotides listed in Table 1 (final primer concentration 0.8 µM). PCR amplification took place in a thermocycler (type 2400, Applied Biosystems, Rotkreuz, Switzerland) using the following conditions: 15 min at 95°C for activation of Taq polymerase, 30 cycles of denaturation at 94°C for 30 s, annealing at a

temperature according to the used primer pair (Table 1) for 30 s and extension at 72°C for 45 s.

Amplified fragments were controlled by agarose gel electrophoresis and analyzed under UV (λ= 254nm). Bands of the expected size were cut and purified with Qiaquick Extraction Kit (Qiagen).

primer	sequence (5'→3')	target	gene position	amplicon size (bp)	annealing temperature (°C)
*Mpneu_eno*_1	ATGAGTGCACAAACTGGAAC	*M. pneumoniae*[1] *eno* (Enolase)	1-20	1371	50
*Mpneu_eno*_2	TTAAGCTTTTTGCGGTTTAAT		1351-1371		
*Mpneu_pdh*_1	ATGTCAAAAACAATTCAA	*M. pneumoniae*[1] *pdh* (Pyruvate dehydrogenase)	1-18	984	50
*Mpneu_pdh*_2	TTACTTTAAAAGTTGGTTAAC		964-984		
*ms_ppa*_fw	GGTATATCCGCACAACTATGG	SG2_*ms*_262[2]	650-670	643	57
*ms_ppa*_rev	TGTCTCGCCTCAGGTGAGAC		1274-1293		
*ms_ppa*_1	ATGTCAAAAAATAATATAGTGGA	*M. suis ppA*[2]	1-23	510	48
*ms_ppa*_2	TTAATAATTTGATTGTTATTCTCC		487-510		

Table 1. Forward and reverse PCR primer for PCR.[1] NCBI accession number: *M. pneumoniae* (NC_000912); [2] nucleotide sequence originated from this study.

3.9 DNA sequencing and sequence analysis

Plasmid DNA was extracted from positive hybridized *E. coli* clones using the GenElute HP Plasmid Miniprep Kit (Sigma) following the manufacturer's instructions.

Customized sequencing of plasmid DNA or purified PCR products was performed by MWG-Biotech (Martinsried, Germany) and 4baselab GmbH advanced molecular analysis (Reutlingen, Germany), respectively. Nucleotide sequences were analyzed using the BLAsTn/BLASTx and the FASTA algorithm (Biocomputing service, University Zürich, www-bio.unizh.ch/).

4. Results

4.1 Screening of *Mycoplasma suis* genomic DNA libraries for enolase and pyruvate dehydrogenase homologous sequences

Previous studies in our laboratory have shown that the two *M. suis* genomic DNA libraries SG1 and SG2 contain a considerable proportion of porcine DNA inserts with an estimated ratio of porcine to *M. suis* DNA of 90:10%. Therefore, an inverse negative selection strategy was performed to separate out library clones enharbouring porcine DNA inserts. By using a colony blot hybridization technique and biotinylated probes directed against whole genomic procine DNA, we were able to identify library clones reacting with the pig specific DNA probe on each tested *E. coli* agar plate in a ratio of approximately 95:5 (% porcine DNA reacting clones vs. % porcine DNA negative clones). A total of 600 *E. coli* clones were selected out on the basis of unreactivity with porcine DNA probes. Plasmid DNA was prepared from these clones and was subjected to further analyses by means of Southern blot hybridization using *M. pneumoniae* specific enolase (*eno*) and pyruvate dehydrogenase (*pdh*) probes. A positive hybridizing result with either the *pdh* or the *eno* specific probe was obtained from 30 library clones.

In particular, 9 clones showed a distinct hybridization signal with the *pdh* probe, the other 21 clones hybridized with the *eno* probe (Table 2). Clones hybridizing with the *pdh* or *eno* probe were partially sequenced. Nucleotide sequences analysis revealed that 16 out of the 30 clones contain porcine DNA inserts with a significant BLAST score ([E] < 10^{-5}) and a G+C content of 50 to 55% which is characteristic for mammalian DNA (Mouchiroud and Bernardi, 1993). Nucleotide sequences of the remaining 14 library clones showed a significant BLAST score for bacterial DNA ([E] < 10^{-5}) and were, therefore, subjected to further analysis (Table 2). None of these clones contained *eno* or *pdh* homologous sequences. Seven clones contained identical sequences with the highest homology to 3-*oxoacyl synthase III* of *Anaeromyxobacter dehalogenans*, one clone showed the highest homology to *accC* biotin carboxylase of *Synechococcus elongatus,* one clone with the phenol hydroxylase regulator gene of *Wautersia numadzuensis.*

Five identical clones yielded highest homology to an inorganic pyrophosphatase of *Ureaplasma (U.) parvum* serovar 3 strain ATCC 700970 with an average identity of 59.8% in 513 nt overlap. Results are summarized in Table 2.

Clones	Hybridization signal with	Database match	Analysis of the database match	
			Gene	Bacterial species
SG1ms_8	*eno*[1]	bacterial	3-*oxoacyl synthase* III	*Anaeromyxobacter dehalogenans* CP000251[4]
SG2ms_58	*eno*[1]	pig	n.d.[3]	
SG1ms_102	*pdh*[2]	pig	n.d.[3]	
SG1ms_150	*pdh*[2]	pig	n.d.[3]	
SG1ms_175	*eno*[1]	bacterial	3-*oxoacyl synthase* III	*Anaeromyxobacter dehalogenans* CP000251[4]
SG1ms_179	*eno*[1]	bacterial	3-*oxoacyl synthase* III	*Anaeromyxobacter dehalogenans* CP000251[4]
SG1ms_195	*eno*[1]	bacterial	3-*oxoacyl synthase* III	*Anaeromyxobacter dehalogenans* CP000251[4]
SG1ms_197	*eno*[1]	bacterial	3-*oxoacyl synthase* III	*Anaeromyxobacter dehalogenans* CP000251[4]
SG1ms_200	*eno*[1]	bacterial	3-*oxoacyl synthase* III	*Anaeromyxobacter dehalogenans* CP000251[4]
SG2ms_211	*pdh*[2]	pig	n.d.[3]	
SG2ms_222	*eno*[1]	pig	n.d.[3]	
SG2ms_223	*pdh*[2]	pig	n.d.[3]	
SG2ms_225	*pdh*[2]	pig	n.d.[3]	
SG2ms_226	*eno*[1]	bacterial	*ppA* inorganic pyrophosphatase	*Ureaplasma parvum* AAF30724[4]
SG2ms_242	*pdh*[2]	pig	n.d.[3]	
SG2ms_250	*eno*[1]	pig	n.d.[3]	
SG2ms_251	*eno*[1]	*Ureaplasma*	*ppA* inorganic pyrophosphatase	*Ureaplasma parvum* AAF30724[4]
SG2ms_252	*eno*[1]	pig	n.d.[3]	
SG2ms_259	*eno*[1]	pig	n.d.[3]	
SG2ms_262	*eno*[1]	*Ureaplasma*	*ppA* inorganic pyrophosphatase	*Ureaplasma parvum* AAF30724[4]
SG2ms_294	*eno*[1]	pig	n.d.[3]	
SG2ms_301	*eno*[1]	*Ureaplasma*	*ppA* inorganic pyrophosphatase	*Ureaplasma parvum* AAF30724[4]
SG2ms_308	*pdh*	pig	n.d.[3]	
SG2ms_322	*eno*[1]	pig	n.d.[3]	
SG2ms_323	*eno*[1]	*Ureaplasma*	*ppA* inorganic pyrophosphatase	*Ureaplasma parvum* AAF30724[4]
SG1ms_351	*eno*[1]	bacterial	3-*oxoacyl synthase* III	*Anaeromyxobacter dehalogenans* CP000251[4]
SG2ms_389	*eno*[1]	pig	n.d.[3]	
SG2ms_450	*pdh*[2]	bacterial	*accC* biotin carboxylase	*Synechococcus elongatus* U59234[4]
SG1ms_464	*pdh*[2]	bacterial	phenol hydroxylase regulator	*Wautersia numadzuensis* AB177762[4]
SG1ms_545	*eno*[1]	pig	n.d.[3]	

Table 2. Nucleotide sequence analysis against data bank entries of 30 M. suis-library clones.

1, *eno*, *M. pneumoniae* enolase specific DNA probe; 2, *pdh*, *M. pneumoniae* pyruvate dehydrogenase specific DNA probe; 3, n.d., not done; 4, Accession number.

4.2 Characterization of the SG2ms_262 *Mycoplasma suis* library clone and identification of the *Mycoplasma suis* inorganic pyrophospatase gene

One representative of the group of identical clones (SG2ms_262) containing inorganic pyrophosphatase (*ppA*) sequences was completely sequenced. To achieve the complete sequence, the SG2ms_262 was sequenced from both vector sides. This sequencing approach provided 800 to 900 bp from each side. The outstanding sequence data were obtained by a subcloning technique using a *BamH*I cleavage site within the DNA insert and the corresponding *BamH*I site in the multiple cloning site of the plasmid pUC18. The cloning strategy is shown in Figure 6A. The SGms_262 plasmid DNA was cut with *Bam*HI and the remaining plasmid DNA lacking the *BamH*I fragment was self-ligated and subjected to sequencing.

Subsequent sequence analysis revealed that the library clone SG2ms_262 contains a 2059 bp insert with an average G+C content of 30.11%. Clone SGms_262 includes two complete open reading frames (ORF): one ORF of 495 bp which shows the highest homology to *U. parvum* inorganic pyrophosphatase *ppA* (63.32%) and another ORF of 375 bp which shows the highest homology to thioredoxin *trx* (44.55%) of *U. parvum*. Figure 6B shows the location and ORF organization of the library clone SGms_262.

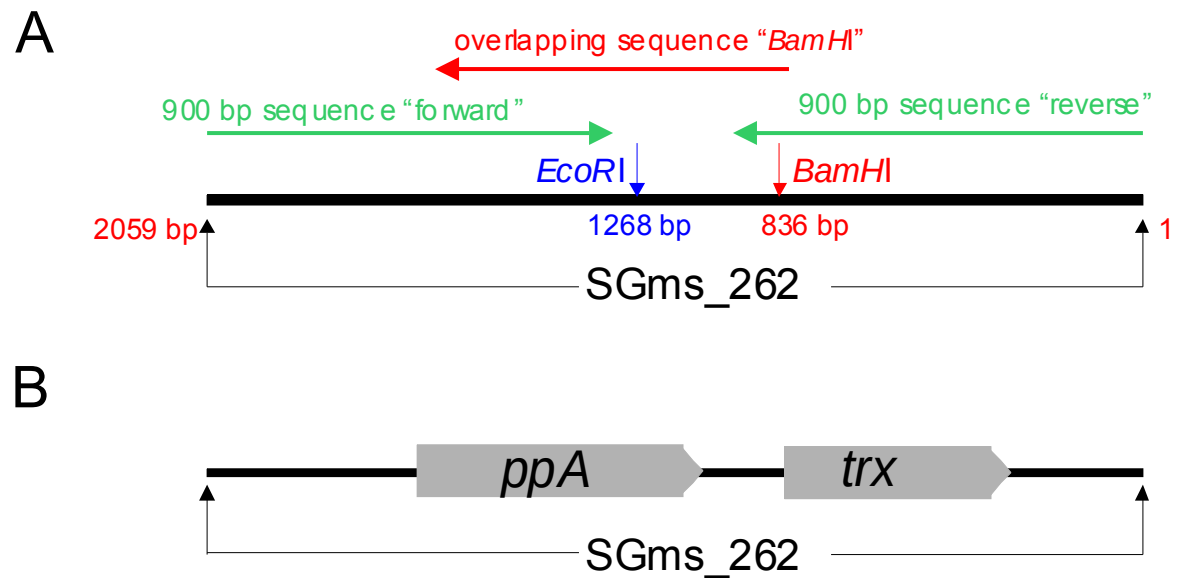

Figure 6. A, Sequencing approach of library clone SG2ms_262 by using a subcloning strategy. Localization of the *BamH*I and *EcoR*I cleavage sites are indicated by arrows. B, Localization of two putative ORFs on the library clone SG2ms_262. *ppA:* putative *M. suis* inorganic pyrophosphatase; *trx*: putative *M. suis* thioredoxin.

To confirm the *M. suis* specificity of SG2ms_262, Southern blot analyses of *M. suis* genomic DNA (*EcoR*I digested) probed with the 2.059 kb SG2ms_262 library fragment were performed. SG2ms_262 hybridized with two genomic *M. suis* fragments of 1.2 and 2.7 kb, respectively (Figure 7). Using biocomputing tools (mapsort) the corresponding *EcoR*I cleavage site could be located on position 1268 within the 2.059-kb insert of SG2ms_262 (Figure 6).

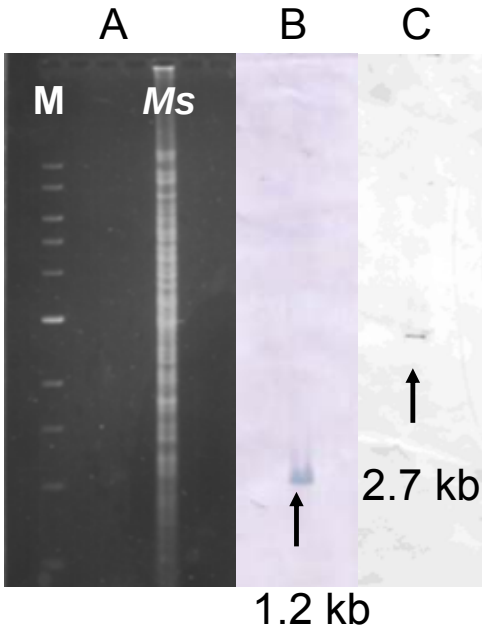

Figure 7. Southern blot hybridization of *EcoR*I digested *M. suis* DNA using DIG labeled SG2ms_262 specific probes. A, Agarose gel electrophoresis, M: molecular weight standard, *Ms*: *M. suis* DNA, *EcoR*I digested. B, C two blots probed with SG2ms_262 specific probes. Hybridizing bands are indicated by arrows.

4.3 Characterization of the *Mycoplasma suis* inorganic pyrophospatase

The identified *M. suis* gene *ppA* is 495 bp in size (Figure 8A). The overall degrees of identity to *ppA* of *U. parvum*, *M. penetrans*, *M. pneumoniae*, and *M. hyopneumoniae* were calculated to be 55.49%, 56.52%, 52.76%, and 53.86% respectively. The *M. suis ppA* translates into a protein of 164 amino acids with a predicted molecular mass of 18.62 kDa and an isoelectric point of 4.72 (Figure 8B). The characteristic prosite signature of the inorganic pyrophosphatase, which is essential for the hydrolysis of pyrophosphate, was identified in the *M. suis* inorganic pyrophosphatase at amino acid positions 54 to 60 using the program predict protein

(http://cubic.bioc.columbia.edu/predictprotein/; Table 3). Figure 9 shows a multiple alignment of the amino acid sequences of the *M. suis* inorganic pyrophosphatase with inorganic pyrophosphatase of different *Mycoplasma* spp. and *U. parvum*.

A

```
atgtcaaaaaataatatagtggagtgctttatagaaattgcaaagcactccaatcttaaa
M  S  K  N  N  I  V  E  C  F  I  E  I  A  K  H  S  N  L  K
tatgagtgtgttgatggcaaattgaaactagatagagtactatttggttcaatggtatat
Y  E  C  V  D  G  K  L  K  L  D  R  V  L  F  G  S  M  V  Y
ccgcacaactatggatatatttctgatactctagcagaggatggagatcctctagatgta
P  H  N  Y  G  Y  I  S  D  T  L  A  E  D  G  D  P  L  D  V
gtagtgctatctaatttctctgtaactccaggaacttatttggattgcaaaattcttggt
V  V  L  S  N  F  S  V  T  P  G  T  Y  L  D  C  K  I  L  G
tctctagaaatggtagattctggagaacaagattgaaaagttattgcaattatggatgcg
S  L  E  M  V  D  S  G  E  Q  D  W  K  V  I  A  I  M  D  A
gatccaagactcaagcatataaattctctagatgatgttcctcaacattgaattgctgaa
D  P  R  L  K  H  I  N  S  L  D  D  V  P  Q  H  W  I  A  E
ttgagaaacttttttgaaagttacaagcaactagagaataagaaagtttcccttggaaac
L  R  N  F  F  E  S  Y  K  Q  L  E  N  K  K  V  S  L  G  N
tttatctctctagaatcaactctttctttaattgaagaatcaaaagctagatgaagaact
F  I  S  L  E  S  T  L  S  L  I  E  E  S  K  A  R  W  R  T
caaggggagaataa
Q  G  G  E  -
```

B

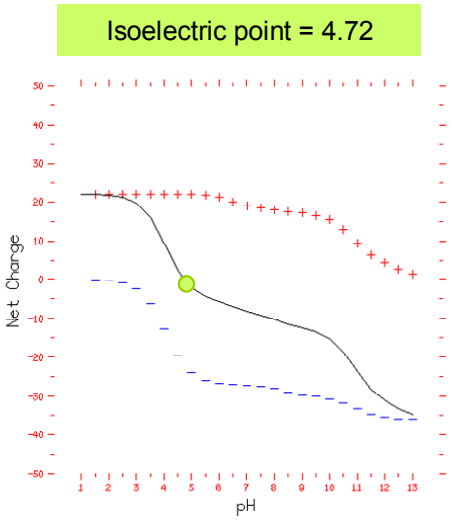

Figure 8. A, Nucleotide and amino acid sequence of *M. suis* inorganic pyrophosphatase. The typical prosite signature was indicated in yellow. B, Analysis of the isoelectric point of the *M. suis* inorganic pyrophosphatase.

Possible signatures for inorganic pyrophosphatases	D[SGDN]D[PE][LIVMF]D[LIVMGAC]
Signature of the *M. suis* inorganic pyrophosphatase (aa position 54 to 60)	DGDPLDV

Table 3. Prosite signature of the *M. suis* pyrophosphatase

```
M. penetrans            ----MKLNVTIEIPKKSNVKYEYDRKTNQISVDRILFGTEVYPHNYGFIKEALDWDGDEL 56
Ureaplasma parvum       ----MKLNVTIEIPKNSNIKYEYDRATKEIMVDRILHGSMVYPHNYGFLKEALDYDGDEL 56
M. pneumoniae           -MDKFLIDVTVEIPKSSKIKYEYDRKTSQIRVDRILFGSESYPQNYGFIANTLDWDGDEL 59
M. genitalium           -MDKFLIDVIVEIPKNSKIKYEYDRQTGQIRVDRILFGSESYPQNYGFIKNTLDWDGDEL 59
M. mobile               -MNEKIIEVNIEIPINSNIKYEFDRSKNKIVVDRILRGDFKYPANYGYVAEALDWDGDEL 59
M. pulmonis             -MSK--IVVNIEIQKDSNIKYEYDRKRGKIVVDRILRGDFRYPANYGYLEDALDWDGDEL 57
M. mycoides             -MKNNVISMVVEIPKGSSNKYEVDEKTKRIKLDRVLYGANFYPGEYGMIENTLDWDGDPL 59
M. capricolum           -MKNNVISMVVEIPKGSSNKYEVDEKTKRIKLDRVLYGANFYPGEYGMIENTLDWDGDPL 59
M. suis                 MSKNNIVECFIEIAKHSNLKYECVDG--KLKLDRVLFGSMVYPHNYGYISDTLAEDGDPL 58

M. penetrans            DALVIADQSFLPGIIVPAKIIGAMEMIDDGETDTKLISVIDCDPRYKHINNLSDLGEHTL 116
Ureaplasma parvum       DVLVFADQAFQPGIKVPARVLGAMKMIDGGETDTKLLAVIDVDPRYKHINNFKDIPLHWL 116
M. pneumoniae           DCFIFADQAFLPGVVVPTRIVGALEMVDDGELDTKLLGVIDCDPRYKEINSVNDLPKHRV 119
M. genitalium           DCFIFADQPFLPATVVPTRIVGALEMIDDGEIDTKLLGVIDCDPRYKEINQISDLPKHRI 119
M. mobile               DVLVYSSETFVPGSLLRARLVGAMKMIDQGETDTKLIAVHADDYRLDKIKELVDIPKEWL 119
M. pulmonis             DVLVYSQEKFLPGTSLNARIVGAMKMIDDGETDTKLIAVHDDDYRLENIKALEDLDSKWL 117
M. mycoides             DVISLCTYPTLPGVEVNVRILGSIKMVDAGEVDTKLFGVFNDDPRFKEYQTLNDVPKHYR 119
M. capricolum           DVISLCTYPTLPGVEVDIRILGSIKMVDAGEVDTKLFGVFNDDPRFKEYQTLNDVPKHYR 119
M. suis                 DVVVLSNFSVTPGTYLDCKILGSLEMVDSGEQDWKVIAIMDADPRLKHINSLDDVPQHWI 118

M. penetrans            KEIQNFFETYKLLQN-KKVVIKGFKDSAWATKEYNECVELMKKYGKMDKDEFVNKMKKEH 175
Ureaplasma parvum       AEVQDFFENYKNLQN-KKVKILGFEDEIWAQKEYEECVLLMKEYGHLKKDEFVTKMMKER 175
M. pneumoniae           DEIIGFLKTYKLLQK-KEVIIKGVQSLEWAKKEYQVCVDLMKQYGKLPKDEFIAKMQKLH 178
M. genitalium           EEILIFLKTYKLLQK-KTVIIKGLKDVCWAKKEYEICLQLMKDYGHLSKDQFIQKMQILH 178
M. mobile               RNVEYFFTNYKNWKGVNQVKINGFEGLKYALAEYEECVHQMEKYGKMPKSEYVKQMQKKH 179
M. pulmonis             EEIKYFFSNYKNWKRPGITKVSGFENTSWALKEYQECKDLMREYGHLPKKEFISKMMKMH 177
M. mycoides             DEIENFFLQYKALQN-KVVKINGWGTLDEALEELEECKSRFEEYKDRLAKGQKDQILAEW 178
M. capricolum           DEIENFFLQYKALQN-KVVRINGWGTLDEALEELEECKARFEEYKDRLAKGQKDQILAEW 178
M. suis                 AELRNFFESYKQLEN-KKVSLGNFISLESTLSLIEESKAR------------------W 158

M. penetrans            PEKYKA-- 181
Ureaplasma parvum       PEKYIK-- 181
M. pneumoniae           PEHYQK-- 184
M. genitalium           PEHYQK-- 184
M. mobile               PEKYTI-- 185
M. pulmonis             PEKYEI-- 183
M. mycoides             KEKGLGQA 186
M. capricolum           KEKGLGQA 186
M. suis                 RTQG-GE- 164
```

Figure 9. Amino acid multiple alignment of the *M. suis* inorganic pyrophosphatase with different *Mycoplasma* spp. and *U. parvum*. The conserved prosite signature was indicated in yellow, amino acids identical with *M. suis* inorganic pyrophosphatase are indicated in green.

4.4 Conservation of *ppA* in different *Mycoplasma suis* isolates

To analyze the presence and conservation of *ppA* in different *M. suis* isolates, a PCR was performed using a primer pair spanning the entire *M. suis ppA* gene. 9 of 10 blood samples from *M. suis* positive pigs revealed an amplicon of the expected size of 510 bp (Figure 10), whereas the blood samples of *M. suis* negative pigs yielded no amplicons. Sequence analysis of the PCR amplicons showed a 100% identity to the SG2ms_262 enclosed *ppA*.

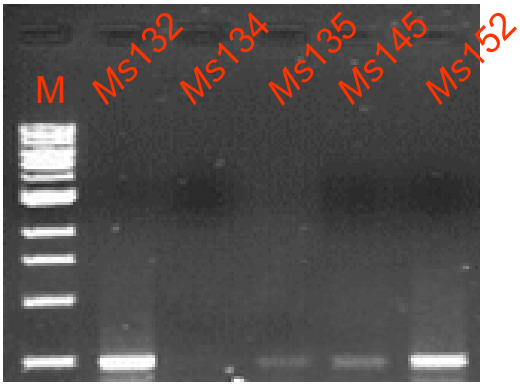

Figure 10. Agarose gel electrophoresis of *ppA* PCR amplicons of 5 different *M. suis* isolates (*Ms*132, *Ms*134, *Ms*135, *Ms*145, *Ms*152).

5 Discussion

To date little is known on the genome of M. suis – as is generally on the genomes of hemotrophic mycoplasmas. The crucial barrier in systematic genetic analyses of *M. suis* is the lack of an *in vitro* cultivation system being the source for sufficient amounts of purified DNA. To date starting material for analyzes of the *M. suis* genome are rather limited stocks of *M. suis* cells purified from porcine blood. This indispensable procedure requires facilities to infect pigs and to purify *M. suis* from the peripheral blood taken during clinical attacks of acute disease.

By primer amplification targeting highly conserved regions of the procaryotic 16S rDNA the nucleotide sequences of the 16S rRNA gene of several hemotrophic mycoplasmas were established.

Phylogenetic classification of the hemotrophic mycoplasmas was thus made possible. Additionally, gene fragments enabling the specific and sensitive diagnostic PCR assays for different species of hemotrophic mycoplasmas were identified.

Nevertheless, the molecular basis of the *M. suis* pathobiology still remains an unresolved contemporary issue.

A promising approach to elucidate the genetic basis of *M. suis* proteins which are probably important pathogenesis factors as well as vaccine candidates or diagnostic antigens was published recently by our laboratory (Hoelzle et al., 2007a,b). By means of serological proteome analysis (SERPA), using sera from experimentally infected pigs, our group has recently described eleven *M. suis*-specific immunoreactive proteins. One protein was characterized by PMF-MALDI-TOF as enolase and one protein as pyruvate dehydrogenase (PDH, Hoelze et al., 2006). Both enzymes are recognized as virulence factors in other bacterial pathogens. In particular, enolase, an enzyme with glycolytic activity, belongs to a class of multifunctional surface proteins with binding properties. For example, in group A streptococci enolase is described as a plasminogen binding protein, and enolase of Staphylococci binds to laminin (Pancholi, 2000). Noteworthy, in the human pathogen *M. pneumoniae* which is phylogenetically closely related to *M. suis* and which exhibits similar pathobiological properties i.e. cold agglutinin induction and adhesion to erythrocytes, PDH is part of the adhesion complex which mediates the attachment to mucosal cells (Dallo et al., 2002).

Therefore, both proteins may also play a role in the host-pathogen-interactions of the *M. suis*-infections in pigs. The topic of this study was to investigate the genetic background of these two proteins found in *M. suis*. Since *M. suis* is not cultivable and the *M. suis* genome has not been sequenced so far we applied a cross-species identification approach: two *M. suis* genomic DNA libraries SG1 and SG2 were screened for homologous DNA sequences using DNA probes specific for the enolase- and PDH gene of *M. pneumoniae*.

One problem which has to be handled in this work was the high percentage of clones with porcine DNA inserts found in the *M. suis* libraries. Preceeding tests gave evidence for a ratio of approx. 90% porcine DNA inserts to approx. 10% inserts of bacterial (*M. suis*) origin. In order to increase the low possibility to detect the clones with *M. suis* DNA inserts, we disqualified right from the start clones carrying porcine DNA inserts by applying an adequate selection strategy. Based on the negative hybridization reaction using total porcine DNA probes a total of 600 clones was selected for further analysis. After hybridization with both the enolase and PDH probe, a sample of 30 clones remained. These clones were thought to carry the

searched homologous *M. suis* DNA sequences insert sought since they showed a positive hybridization reaction with the DNA probes directed against the enolase and PDH gene of *M. pneumoniae*.

Upon verifying the hybridization tests by sequencing the DNA insert of the 30 hybridization positive clones, 16 clones had to be separated as they carried mammalian i.e. porcine DNA. The remaining 14 clones carried bacterial DNA. However, detailed analysis did not proof the origin of the inserts in *eno* or *phd* homologous *M. suis* sequences. Against that, we found evidence for the existence of four other bacterial genes in *M. suis*. The inserts of seven clones showed homologies to a putative 3-oxoacyl synthase III was detected (highest homology with the relevant gene of *Anaeromyxobacter dehalogenans*). The inserts of an other five clones showed homologies to the gene encoding for the inorganic pyrophosphatase of *Ureaplasma parvum* serovar 3 (approximate homology of 59.8% in 513 nt overlap). This fact is of particular interest since this gene belongs to a representative of the *Mcoplasmataceae* family and is thus related to the *M. suis* bacterium. While analysing the inserts of the remaining two clones, in one case we found evidence for the presence of a gene encoding an accC biotin carboxylase with the highest homology to the corresponding gene of *Synechococcus elongatus*. Finally, the insert of the 14th clone related to the gene of a phenol hydroxylase regulator with the highest homology to the corresponding gene of *Wautersia numadzuensis*.

In summary, we have to state that our cross-species identification strategy to detect the encoding genes of the *M. suis* enolase or PDH was not successful. On the other hand, we were nevertheless able to identify four novel DNA sequences which most likely encode for interesting *M. suis* proteins.

At first glance it might seem unusual to detect gene sequences of, for instance, an inorganic pyrophosphatase using an enolase gene probe specific for *M. pneumoniae*. However, in our opinion this result is comprehensible for the following reasons: all five clones harbouring the ppA gene as the insert were hybridized only with the enolase probe. In fact, comparison of the nucleotide sequence of the *M. pneumoniae* enolase gene with the entire ppA insert of the representative clone SG2ms_262 confirmed that the region from nucleotide position 826-1275 reveals an identity of at least 45%, and this level of DNA-homology is sufficient for a positive hybridization under the given reaction conditions. The latter were designed to hybridize DNA of

appr. 40-60% homology which is expected between *M. suis* and *M. pneumoniae*. (Hoelzle et al, 2007a,b). In adddition, we must also consider that our genomic *M. suis* libraries are in all probability not exhaustive. Thus, an indefinable proportion of risk remains that the sample of 600 clones derived from the libraries were not sufficient to detect an enolase or PDH insert. This might be due to the low prevalence of the mentioned clones or due to the lack of clones harbouring the gene encoding for the *M. suis* enolase.

Out of the four novel DNA sequences three yield a significant database match ([E] of $\geq 10^{-5}$) only with bacteria other than *Mycoplasma*. This was to be expected since it is well known that hemotrophic mycoplasmas encode a large number of unique proteins (Messick et al. (2003). Interestingly, the DNA insert of five clones was identified as the gene encoding for an inorganic pyrophosphatase with an overall homology of 63.32% with the respective gene of *Ureaplasma parvum*.

Clone SG2ms_262 was chosen as a representative for further studies since the ppA gene is ubiquitous in nature and plays an important role in the bacterial energy metabolism, providing a thermodynamic pull for biosynthetic reactions such as protein, RNA and DNA synthesis (Kronberg, 1962). According to Peller (1976) nucleic acid syntheses would be energetically impossible *in vivo* if they were not coupled to the hydrolysis of pyrophosphate (PPi), catalyzed by PPase. In addition, various findings suggest that PPase might play an important role not only in the regulation of macromolecular synthesis and growth (Airas et al., 1986) but also in evolutionary events, by affecting the accuracy by which DNA molecules are copied during chromosome duplication (Herbomel et al.; 1980). Furthermore, Spaltmann and co-workers (1999) postulated that PPases are considered excellent targets for antibacterial and antifungal drug development.

Detailed analysis of the novel *M. suis* ppA gene revealed that the ORF for the ppA is 492 bp (495 including stop codon) in length. The deduced amino acid sequence is 164 amino acids in length showing a low G+C content of 30.11%. This G+C value is characteristic for mycoplasmal genes. Whilst using ppA-specific oligonucleotides, ppA-amplicons of 100% identity were amplified from 90% of *M. suis* isolates investigated. We thus conclude that ppA is conserved in *M. suis*.

It is of particular interest to note that the insert of SG2ms_262 harbours a second ORF encoding a putative *M. suis* thioredoxin. The thioredoxin system operates via

redox-active disulphides and provides electrons for a wide range of metabolic processes in prokaryotic cells. Especially within the genus *Mycoplasma* the thioredoxin complex apparently belongs to the metabolic core reactions (Pollack et al., 1997; Wieles et al., 1995).

In view of the fact that apart from the recently described MSG1 und HSP A1, there are no findings on the genetics of *M. suis*, the present study has brought forth interesting facts. The establishment of an *M. suis*-specific ppA gene and a novel ORF indicating a *M. suis* thioredoxin complex are of particular interest. Further studies are now started to carry out functional analyses on borth found new *M. suis* proteins.

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